Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

RHCG suppresses cervical cancer progression through inhibiting migration and inducing apoptosis regulated by TGF-β1

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ARTICLE INFO

Article history:
Received 21 May 2018
Accepted 27 May 2018
Available online xxx

Keywords:
Cervical cancer
RHCG
Migration and apoptosis
NF-κB
TGF-β1

ABSTRACT

Cervical cancer is the second commonest cancer among women in the worldwide, and the majority cause of death in various countries, highlighting the importance of investigating new therapeutic targets. Rh family, C glycoprotein (RHCG) belongs to the Rhesus (Rh) family and was first identified as Rh blood group antigens. It has been confirmed to function in cancer progression, including prostate cancer and esophageal squamous cell carcinoma. However, its role in cervical cancer has never been explored. The present study indicated that RHCG was down-regulated in cervical cancers compared to that in normal cervical tissues, and further decreased in cervical cancer cell lines. Functionally, RHCG overexpression reduced cervical cancer cell proliferation and migration, as evidenced by the decreased transforming growth factor (TGF)-β1, matrix metalloproteinase (MMP)-2 and MMP-9 expressions in cancer cells; however, an opposite effect was observed when RHCG was knocked down. Further, increase of RHCG markedly induced apoptosis in cervical cancer cells by improving the cleavage of Caspase-3 and poly (ADP-Ribose) polymerase (PARP). And cells transfected with RHCG siRNA exhibited a notable reduction of cleaved Caspase-3 and PARP. Moreover, nucleus nuclear factor-κB (NF-κB) and whole cell xIPA expressions were markedly reduced by over-expressing RHCG. Conversely, suppressing RHCG elevated NF-κB activation and xIPA expression in cervical cancer cells. Notably, we found that TGF-β1 treatment could abolish the effects of RHCG over-expression on the reduction of cell migration and enhancement of apoptosis in cervical cancer cells. Over-expressing RHCG-reduced NF-κB activation and xIPA expression were also abrogated by TGF-β1 pre-treatment. Additionally, enhancing NF-κB activity could restore xIPA expressions and decrease apoptotic response in cervical cancer cells over-expressing RHCG. In vivo, we also found that RHCG over-expression reduced cervical tumor growth through the same signaling pathways as we found in vitro. Therefore, RHCG may be a potential prognostic biomarker and therapeutic target for human cervical cancer.

1. Introduction

Cervical cancer remains one of the leading causes of tumor-related deaths worldwide [1]. Although there is accumulating evidence that early detection of cervical cancer has decreased mortality, the prognosis of advanced or recurrent cervical cancer remains very poor [2]. Presently, therapeutic treatments for cervical cancer include surgery with chemotherapy, radiation therapy, and concurrent chemoradiation therapy [3]. Due to the poor prognosis of patients suffering from cervical cancer, targeted therapeutics attracted researchers attention to develop effective treatments of advanced, recurrent, or metastatic cervical cancer.

Rh type C-glycoprotein (RHCG) functions as an electroneutral and bidirectional ammonium transporter [4]. RHCG belongs to Rhesus (Rh) family, which was first identified as Rh blood group antigens in human erythroid cells. Rh proteins are tightly associated with the family of ammonia transporter proteins, characterized by the presence of 12 transmembrane-spanning segments [5]. Hence, Rh family proteins can fall into two functionally distinct groups, including ammonia transporting Rh glycoproteins (RHAG, RHBG, and RHCG) and nontransporting Rh proteins (RHD and RHCE) [6,7]. The nonerythroid Rh glycoproteins, RHBG and RHCG, are ammonia transporters, which are widely expressed in various

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https://doi.org/10.1016/j.bbrc.2018.05.183
0006-291X/

tissues. And RHCG was highly expressed in human skeletal muscle, kidney and oral cavity; however, its physiological functions are not well explained [8,9]. As reported, RHCG was decreased in tongue squamous cell carcinoma and in esophageal squamous cell carcinoma [10,11]. However, the effects of RHCG decrease on tumor growth, including cervical cancer, are still not yet investigated.

In the study, we calculated the expression of RHCG in human cervical cancer tissues and their corresponding non-tumor cervix uteri samples. Functional analysis of cervical cancer cells transfected with RHCG overexpression plasmid or RHCG siRNA were carried out to reveal the role of RHCG in cervical cancer progression. RHCG exhibited anti-tumor effects on cervical cancer, which might be a potential a potential therapy target for the treatment of cervical cancer.

2. Materials and methods

2.1. Patients and information

A total of 51 patients with cervical cancer who received surgery at the Department of Obstetrics and Gynecology, Jinhua Municipal Central Hospital (Zhejiang, China) between January 2008 and December 2014 were included in the study. Their corresponding non-tumor cervix uteri samples were used as controls. Specimens were collected from patients for protein and RNA extraction. No patients received preoperative treatment. Informed consent was obtained from all patients before the collection of specimens. The study was reviewed and approved by the Institutional Review Board at Jinhua Municipal Central Hospital.

2.2. Culture and transfection of cells

Human cervical cancer cell lines, including Siha, Caski, Hela, C4-1, and C-33a, and normal endocervical cell line (End1/E6E7) were obtained from American Type Culture Collection (ATCC, USA). Human cervical cancer cell line of TC-1 was purchased from the Tumor Center of Chinese Academy of Medical Sciences. All cells were routinely maintained in RIP1640 or DMEM medium (Invitrogen, USA) supplemented with 10% FBS (Gibco, USA) at 37 °C in a humidified air atmosphere containing 5% CO_2. For RHGC overexpression plasmid, vector pcDNA3.1 plasmid was obtained from KpnI and EcoRV (TransGen Biotech, china). The code sequence of RHGC amplified using PCR with Quick-Fusion Cloning Kit (Bimake, Houston, USA). The siRNA against RHGC and negative control siRNA were obtained from Generay Co., Ltd (Shanghai, China). Cells were transfected with plasmids with DNA Transfection Reagent (Bimake) or with RHGC siRNA using Lipofectamine™-2000 (Invitrogen) when they grew to 70% confluence following the manufacturer's protocols.

2.3. Cell viability assay

Cell proliferation analysis was performed using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. The results were measured by absorbance at 450 nm.

2.4. Quantitative real time-PCR (RT–PCR) and western blot analysis

Total RNA was isolated using TRIZOL (Invitrogen) and reverse transcribed into cDNA using Prime Script™ RT Master Mix (Takara, Japan). Gene transcripts were quantified using CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with SYBR Premix Ex Taq (Takara) and normalized with GAPDH. All primers are listed in Supplementary Table 1. Ct values were analyzed using comparative threshold cycle method and normalized to GAPDH.

CelLytic™ NuCLEAR™ Extraction Kit (Sigma Aldrich, USA) was used for nuclear protein extraction. Cells or tumor tissue samples were lysed on ice using a cell lysis buffer (125 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1%Triton X-100 and 5 mM EDTA) containing both 1% protease inhibitor and 1% phosphatase inhibitor cocktail (Sigma-Aldrich). The resulting lysates were separated on 10% SDS-PAGE, transferred to a PVDF membrane (Millipore, USA). The membrane was then blocked with 5% skim milk and incubated with primary and secondary antibodies (1:5000, Abcam, USA). The blots were visualized with ECL reagent (Millipore). The primary antibodies were shown as follows: antibody against RHCG (1:500, #sc-100287, Santa Cruz, USA), TGF-β1 (1:1000; #ab92486, Abcam), phosphorylated NF-κB (1:1000; #ab86299, Abcam), NF-κB (1:1000; #ab16502, Abcam), MMP-2 (1:500, #sc-13595, Santa Cruz), MMP-9 (1:500, #sc-13520, Santa Cruz), Caspase-3 (1:1000; #ab13847, Abcam), PARP (1:1000, #9532, Cell Signaling Technology, USA), xIAP (1:500, #sc-55551, Santa Cruz), Lamin B (1:500; #sc-374015, Santa Cruz), and GAPDH (1:1000, #ab8245, Abcam).

2.5. Flow cytometry analysis

After double staining with 5 μL of APC-Annexin V, the cells were then incubated for 15 min at room temperature in the dark followed by the addition of 5 μL of propidium iodide (PI) at room temperature for 5 min in dark. Finally, cells were analyzed uisng a flow cytometer (FACScan; BD Biosciences, USA) equipped with Cellquest software (BD Biosciences).

2.6. Transwell analysis for migration

An 8-μm pore size transwell chamber (Corning, USA) was used for transwell migration assay. A total of 1×10^5 cells in 100 μL medium in the absence of FBS were plated in the upper chamber and 500 μL medium containing 10% FBS was covered on the bottom chambers as chemoattractant. After incubation for 24 h, non-migratory cells on the upper membrane surface were removed, and the cells on the bottom surface were fixed with 4% polyoxymethylene and then stained with 0.1% crystal violet for 10 min. Cancer cells were counted through photographing 5 random fields using a light microscope.

2.7. Immunofluorescent (IF) analysis

Cells were fixed with 4% paraformaldehyde phosphate buffer solution and incubated with antibody against RHCG (1:100, #sc-100287, Santa Cruz), and antibody against NF-κB (1:100, #ab86299, Abcam, USA) at 4 °C overnight. Alexa Fluor 594-conjugated secondary antibodies (Abcam) were incubated at room temperature for 30 min. The nuclei were stained with DAPI (KeyGen Biotech). Then, the images were taken with a fluorescent microscope.

2.8. Cervical tumor xenograft model

Animal experiments were performed according to the guideline for the Regulations for Animal Experiments and Related Activities at Jinhua Municipal Central Hospital. 6-week-old female Balb/c nude mice were purchased from Nanjing Peng Sheng Biological Technology Co Ltd (Nanjing, China). 1.0×10^7 Siha cells stably transfected with OE-Con, OE-RHCG, si-Con or si-RHCG were subcutaneously injected into the flank region of nude mice to establish cervical cancer xenograft model (n = 6/group). Tumor volume was monitored through measuring the longest dimension (length) and shortest dimension (width) using dial calipers at 3-day intervals. Tumor volume was evaluated using the following formula: tumor

Please cite this article in press as: D.-G. Wang, et al., RHCG suppresses cervical cancer progression through inhibiting migration and inducing apoptosis regulated by TGF-β1, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.05.183